Changes in foliar cytokinins of *Salix babylonica* and *Ginkgo biloba* prior to and during leaf senescence

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With leaf maturity, there was a trend towards an increase in the proportion of cytokinin O-glucosides. This may contribute to the onset of senescence. After separation of the ethanolic leaf extracts by Sephadex LH-20 and HPLC, detection of cytokinin-like activity by the soybean callus bioassay illustrated that the two species differed markedly in their pattern of change of cytokinin content with leaf age. Mature leaves of *Salix babylonica* contained high levels of O-glucoside conjugates, which decreased prior to the initiation of chlorophyll loss and during the course of senescence. A similar pattern was observed with the free base cytokinins, mainly zeatin (Z) and isopentenyladenine (iP) in this species. *Gingko biloba*, on the other hand, exhibited an extremely complex qualitative cytokinin profile for mature leaves. Riboside, O-glucoside and O-glucoside riboside derivatives of both zeatin and dihydrozeatin (DHZ) appeared to dominate, although the latter was of greater importance. Senescence was characterized by a decrease in free base forms, Z, DHZ and iP, and a corresponding increase in O-glucosides, ribosides and one unknown compound, right up until abscission. It therefore appears that although cytokinins are implicated in the control of foliar senescence in both species studied, this is achieved by very different mechanisms.

Samehangend met blaar-volwassewording was daar 'n toename in die proporsie van sitokinien O-glukosiede. Dit mag wees as gevolg van die aanvang van veroudering. Na skeiding van die etanoliese blaarekstrakte met Sephadex LH-20 en HPLC is deur middel van die sojaboonkallus-biotoets gevind dat die twee spesies heelwat van mekaar verskil ten opsigte van die patroon van sikokinien verandering met veroudering. Volwasse blare van *Salix babylonica* het hoë vlakke van O-glukosied-konjugate bevat wat verminder het voor die verlies aan chlorofiel en gedurende die verouderingsproses. 'n Soortegelyke patroon is gevind ten opsigte van die vrye sitokiniene, hoofsaaklik zeatien (Z) en isopentanieladenien (iP) in die spesies. Aan die ander kant het *Gingko biloba* 'n baie komplekse kwalitatiewe profiel vir volwasse blare getoon. Ribosied, O-glukosied en ribosied-O-glukosied derivate van beide zeatien en dihidrozeatien (DHZ) was dominant, met laasgenoemde van groter belang. Veroudering is gekarakteriseer deur 'n afname in die sitokinien-basisse, Z, DHZ en iP, en 'n gelyktydige toename in O-glukosiede, ribosiede en een onbekende verbinding, tot en met blaarafsnyding. Dit wil voorkom asof alhoewel sitokiniene in beide spesies in die beheer van blaarveroudering geïmpliseer is, die proses deur verskillende meganismes voltrek word.

Keywords: Cytokinins, *Gingko biloba*, leaf senescence, *Salix babylonica*.

Introduction

Plant senescence is the result of a complex series of processes requiring co-ordination with relevant environmental stimuli and correlative control within the plant as a whole. As plant growth regulators have long been regarded as long-distance messengers transducing environmental stimuli into appropriate developmental responses (Cleland 1983), it is logical that these compounds may play a role in co-ordinating changes associated with senescence. Evidence to support this theory with respect to leaf senescence was first supplied by Richmond & Lang (1957) who demonstrated that application of the synthetic cytokinin, kinetin, to excised leaves of *Xanthium strumarium* delayed senescent processes such as chlorophyll loss. This, and other studies using exogenously applied cytokinins, led to the development of a hypothesis that the cytokinin content of a leaf is one of the most important factors governing the onset of the senescence syndrome.

The effects of an exogenously applied hormone, however, do not necessarily mirror the endogenous situation within the leaf. This observation led to many workers investigating the changes in endogenous cytokinin concentrations with leaf age, and attempting to relate this to the control of foliar senescence. Studies of this nature have, however, yielded conflicting results. Hewett & Wareing (1973a, 1973b) investigated the changes in foliar cytokinin content of *Populus* × *robusta* during development and produced results consistent with the hypothesis that a decrease in cytokinin content coincides with the onset of senescence. It was illustrated that the total amount and activity of cytokinins was at a maximum in expanding leaves, decreasing with leaf age. A similar situation was reported by Renfroe & Brown (1983), where endogenous cytokinin concentrations in *Platanus occidentalis* were highest in small leaves and decreased to a quarter of the original levels as the leaves enlarged. Most recently, Singh *et al.* (1992a) demonstrated that pre-senescent leaves of *Nicotiana rustica* contain three times more of the free base zeatin, than that of their partly senescent counterparts.

Studies that show this decrease in overall cytokinin activity with leaf age are, however, few. Many workers reported that total cytokinin activity actually increased with leaf age in many plant species (Lorenzi *et al.* 1975; van Staden 1977; Henson 1978a, 1978b; van Staden & Davey 1978; Ilan & Goren 1978; Hendry *et al.* 1982). As this appeared to refute the hypothesis that foliar senescence is triggered by a decrease in cytokinin concentrations, workers began to investigate the qualitative changes in foliar cytokinin content in more detail. Hewett & Wareing (1973b, 1973c) illustrated that only one cytokinin was present in senescing leaves of *Populus* × *robusta*. Evidence suggested that this was a glucoside. Using this observation, van Staden (1976a,

1977) illustrated that although cytokinins appear to accumulate with age in the leaves of both *Salix babylonica* and *Ginkgo biloba* respectively, this was due to an increase in the proportion of compounds susceptible to cleavage by β-glucosidase, namely cytokinin O-glucosides. Subsequently, numerous research groups have demonstrated that not only are these O-glucoside conjugates important components of leaf tissue in many species (Henson & Wareing 1976; Wang *et al.* 1977; van Staden 1976b, 1978; Davey & van Staden 1978a, 1978b; van Staden *et al.* 1983; Nagar & Saha 1985; Hansen *et al.* 1988; Saha & Sirca 1990), but that they show a tendency to accumulate with leaf age and senescence (Lorenzi *et al.* 1975; van Staden 1976a; van Staden & Davey 1978; Hendry *et al.* 1982; van Staden *et al.* 1983; Saha & Sirca 1990). Interestingly, Peters & Beck (1992) have recently reported a similar situation in cell suspension cultures of *Chenopodium rubrum* where O-glucosides are the dominant form of cytokinin present in the stationary phase of growth, although they are also present in fairly high concentrations during the logarithmic growth period.

The results of these investigations therefore lead to the question as to whether endogenous cytokinin concentrations do indeed play a role in controlling leaf senescence. Intimately related to this is the possible role of cytokinin O-glucosides within the plant system. As these compounds represent the form of cytokinin whenever accumulation occurs (Letham & Palni 1983), it has been hypothesized that they may represent inactivation products of free base conjugation (McGaw & Horgan 1985). Therefore an increase in O-glucoside content could be linked to a decrease in the active pool of cytokinins within the leaf, thus triggering senescent processes. It has even been suggested that the actual accumulation of O-glucosides themselves may be involved in this initiation (van Staden *et al.* 1988). Considering the equation:

Free base of cytokinin + glucose ↔ cytokinin O-glucoside

it is possible that this flux is in equilibrium in mature, but non-senescing leaves. At the onset of senescence, however, the concentration of free base decreases, either from reduced supply or decreased biosynthesis (Singh *et al.* 1992b). To re-establish the equilibrium, there will temporarily be a nett conversion of cytokinin O-glucoside to the compounds on the left. After attainment of equilibrium this will result in a different ratio of free base:glucoside. It may be this shift in the flux of cytokinins between the free base and O-glucoside pools that acts as a trigger to senescence processes, rather than alterations in bulk concentrations. Most of the studies investigating qualitative and quantitative changes in foliar cytokinin content with leaf age have utilized representative material of expanding, mature and senescing leaves, or at best, monthly samples. Analysis of the cytokinin content of such widely spaced samples would not detect small changes in the flux between free base and O-glucoside which may coincide with the onset of the senescent syndrome. Studies are therefore required where leaf samples are collected more frequently during the transition from the mature to the senescence state in order to examine this aspect in more detail.

The equation illustrated above shows that flux between the O-glucoside and free base pools is bidirectional. This is due to evidence that the conjugation of glucose to the free base is a reversible process (van Staden & Papaphilippou 1977). This possible hydrolysis of the conjugate introduces the possibility that O-glucosides may not only represent stable end products of cytokinin inactivation, but may be re-used when required and therefore represent putative storage forms. On relating this to foliar senescence in deciduous trees, however, one major problem is encountered. As compounds present in the leaf on abscission are lost to the plant, the O-glucosides would have to be exported prior to leaf abscission to represent true storage products. Therefore in order to investigate the role of these compounds with respect to foliar senescence and the cytokinin content of the deciduous plant as a whole, it is necessary to investigate their concentration in leaves prior to and during senescence right up until the point of abscission.

This study reports on the endogenous cytokinin content of leaves of two deciduous trees: the angiosperm *Salix babylonica* and the gymnosperm *Ginkgo biloba* from late summer (April) to early winter (June).

Materials and Methods

Plant material

Fresh leaves of *Salix babylonica* L. and *Ginkgo biloba* L. were collected at two-weekly intervals from mid-April to the completion of leaf abscission (June). The final sample of leaves of *Ginkgo biloba* (post-abscission) was collected from freshly abscinded leaves beneath the tree. Samples were collected at the same time each day to minimize any interference by daily fluctuations. After collection, leaves were weighed, flash frozen in liquid nitrogen, freeze-dried and stored in a deep-freeze until required.

Extraction and analysis for chlorophyll

Three grams of freeze-dried leaf material was homogenized in a mortar and pestle and extracted overnight in 20 ml ice-cold methanol (Holden 1965). The resultant homogenate was filtered through Whatman No. 1 filter paper and the residue rinsed with a further 10 ml methanol. The absorbance of aliquots of the extracts was then measured spectrophotometrically, using a Beckman DU-65 Spectrophotometer at wavelengths 470 nm, 652 nm and 665 nm. Absolute chlorophyll concentrations (chlorophyll *a* and chlorophyll *b*) were then calculated according to the equations of Lichtenthaler (1987).

Extraction and analysis for cytokinins

Freeze-dried leaf tissue was homogenized in a mortar and pestle with 80% ethanol and left to extract overnight with continuous agitation. Homogenates were then filtered, rinsed, reduced to dryness *in vacuo* at 37°C and resuspended in 4 ml 80% ethanol for further analysis. This procedure is outlined in Figure 1.

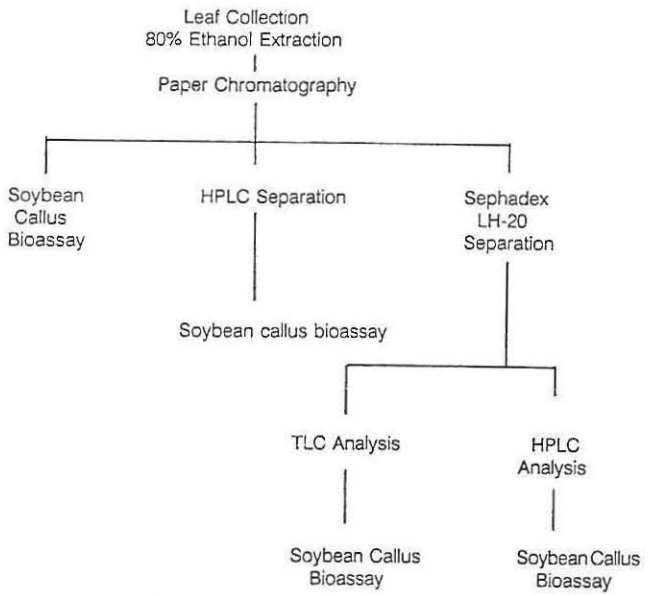

Figure 1 Procedure of analysis of leaf extracts of *Salix babylonica* and *Ginkgo biloba*.

Paper chromatography

Aliquots of plant extract were strip-loaded on to Whatman No. 1 chromatography paper and separated using a solvent system of isopropanol:25% ammonium hydroxide:water (10:1:1 v/v). The chromatograms were dried overnight at 35°C and stored in plastic bags in a deep-freeze until required for analysis.

Fractions separated by paper chromatography were either assayed for biological activity or eluted for further analysis. Elution was achieved using an 80% ethanol extraction overnight, with continuous shaking. The eluent was filtered under vacuum through two layers of Whatman No. 1 filter paper and the paper residue was washed with twice the initial volume of 80% ethanol. The combined eluents were then reduced to dryness *in vacuo*, and resuspended in 80% ethanol for further analysis.

Column chromatography

Plant extracts were loaded onto a 2.5 × 90 cm Sephadex LH-20 column and eluted with 35% redistilled ethanol at a flow rate of 15 ml h^{-1} (Armstrong *et al.* 1969). Authentic cytokinin standards were run using this system in order to establish their separation and relative retention volumes. Fractions of 40 ml were collected and dried in air prior to bioassay or further analysis.

Thin-layer chromatography (TLC)

Plant extracts were strip-loaded onto a 20 × 20 cm UV fluorescent $60F_{254}$ silica gel plate (Merck), 5 cm from the edge. For separation of cytokinin free bases from their ribosides and glucosides, a solvent system of the upper phase of *n*-butanol:25% ammonium hydroxide: water (6:1:2 v/v) was used. For the separation of different cytokinin groups, for example Z derivatives from iP derivatives, a solvent system of *n*-butanol:acetic acid:water (12:3:5 v/v) was employed. After separation, the plates were dried and the relevant fractions scraped from the plate for bioassay.

High-performance liquid chromatography (HPLC)

The HPLC system used comprised a Varian 5000 Liquid chromatograph and a Varian UV 50 variable wavelength detector, set at 254 nm. For analysis of endogenous cytokinins, a reverse-phase semi-preparative Hypersil 5 ODS 25 cm × 10 mm column was used, at a solvent flow rate of 3 ml min^{-1}. The column was eluted over 90 min with a 5–50% methanol gradient in 0.2 M acetic acid buffered to pH 3.5 with triethylamine (Lee *et al.* 1985). Separation of authentic cytokinin standards using this system is illustrated in Figure 2. If required for further analysis, fractions were collected at 1-min intervals and air-dried.

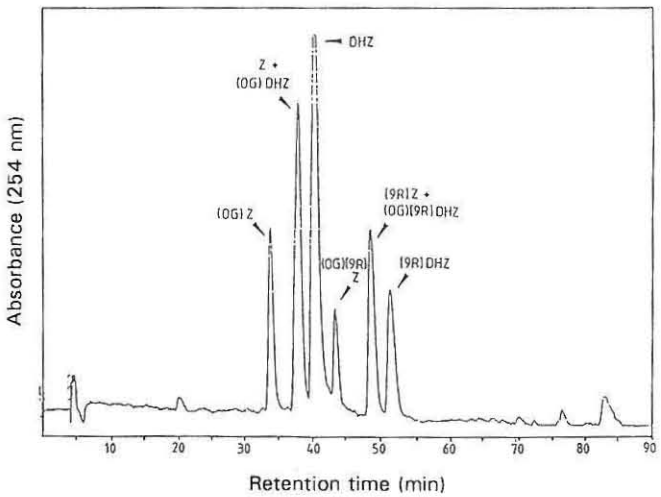

Figure 2 HPLC trace showing the separation of authentic cytokinin standards. Column: reverse phase, semi-preparative Hypersil 5 ODS 25 cm × 10 mm. Methanol gradient 5–50% with 0.2 M acetic acid (pH 3.5 with triethylamine). Flow rate 3 ml min^{-1}.

β-Glucosidase treatment

Dried extracts were resuspended in 1 ml 0.05 M sodium acetate buffer (pH 5.0) containing 1 mg almond β-glucosidase (Sigma Chemical Company, Germany). The enzyme represents 5.5 units per mg of solid, where one unit liberates 1 μmole of glucose from salicin per min at pH 5.0 and 37°C. The extract was then incubated at 37°C for 3 h (Henson 1978a) after which the reaction was terminated by the addition of 1 ml absolute ethanol. The resultant hydrolysate was filtered and evaporated prior to subsequent analysis.

Potassium permanganate ($KMnO_4$) treatment

Dried extracts were resuspended in 1 ml distilled water. A 0.1% aqueous solution of $KMnO_4$ was added drop-wise to the sample until the purple colour remained. The sample was left to stand at room temperature for 6 min. After addition of 1 ml absolute alcohol, precipitation of the $KMnO_4$ allowed for its separation from the sample by filtration. The sample was then filtered and evaporated prior to subsequent analysis (Miller 1965).

Soybean callus bioassay

In all cases biological activity and therefore presence of cytokinins was estimated using the soybean callus bioassay (Miller 1965). All extractions and bioassays were repeated at least twice. The average of the results obtained are presented in the figures.

Results

Changes in endogenous cytokinins of leaves of *Ginkgo biloba* with foliar senescence

Separation of ethanolic extracts of leaves of *Ginkgo biloba* by Sephadex LH-20 showed a trend towards a qualitative decrease in cytokinin content from mature leaves to senescent leaves, and finally abscinded leaves (Figure 3A, B, C & D). The results illustrated do not represent the entire sampling period, but rather, the time when change was occurring. Samples prior to and including 12 May (Figure 3A) were very similar. The observed trend favoured the accumulation of compounds co-chromatographing with O-glucoside and riboside derivatives. In earlier samples (12 May and 26 May) riboside derivatives appeared to predominate, with the O-glucoside derivatives increasing by 9 June (Figure 3C). Free base cytokinins in the form of both zeatin and iP derivatives decreased from 12 May, with negligible amounts present in the leaves collected from beneath the tree on 25 June. Chlorophyll concentrations were found to be approximately 800 μg g^{-1} FW in mature leaves of *Ginkgo biloba* and decreased rapidly after 12 March to basal levels in abscinded leaves (Figure 4).

In mature non-senescing leaves of *Ginkgo biloba* (Figure 3A) the cytokinin complement after separation by Sephadex LH-20 was represented by seven major peaks of activity. Five of these co-eluted with authentic cytokinin standards. The large peak of activity, eluting at a volume of 520 ml, did not co-elute with any standards on Sephadex LH-20, but on separation by TLC, it was found to have chromatographic properties similar to the cytokinin free bases, zeatin and dihydrozeatin [Figure 3A(i)]. The second-largest peak of activity was found to co-elute with authentic (OG)Z (elution volume 240 ml). On further analysis by TLC, this peak was shown to be composed of two separate regions of activity, co-eluting with (OG)Z and [9R](OG)Z [Figure 3A (ii)]. Treatment with β-glucosidase resulted in a shift in activity to co-elute with Z and [9R]Z respectively. These cytokinins cannot be resolved from their dihydro derivatives by paper chromatography. HPLC analysis of this fraction, however, revealed that the detected activity co-eluted with (OG)DHZ, as

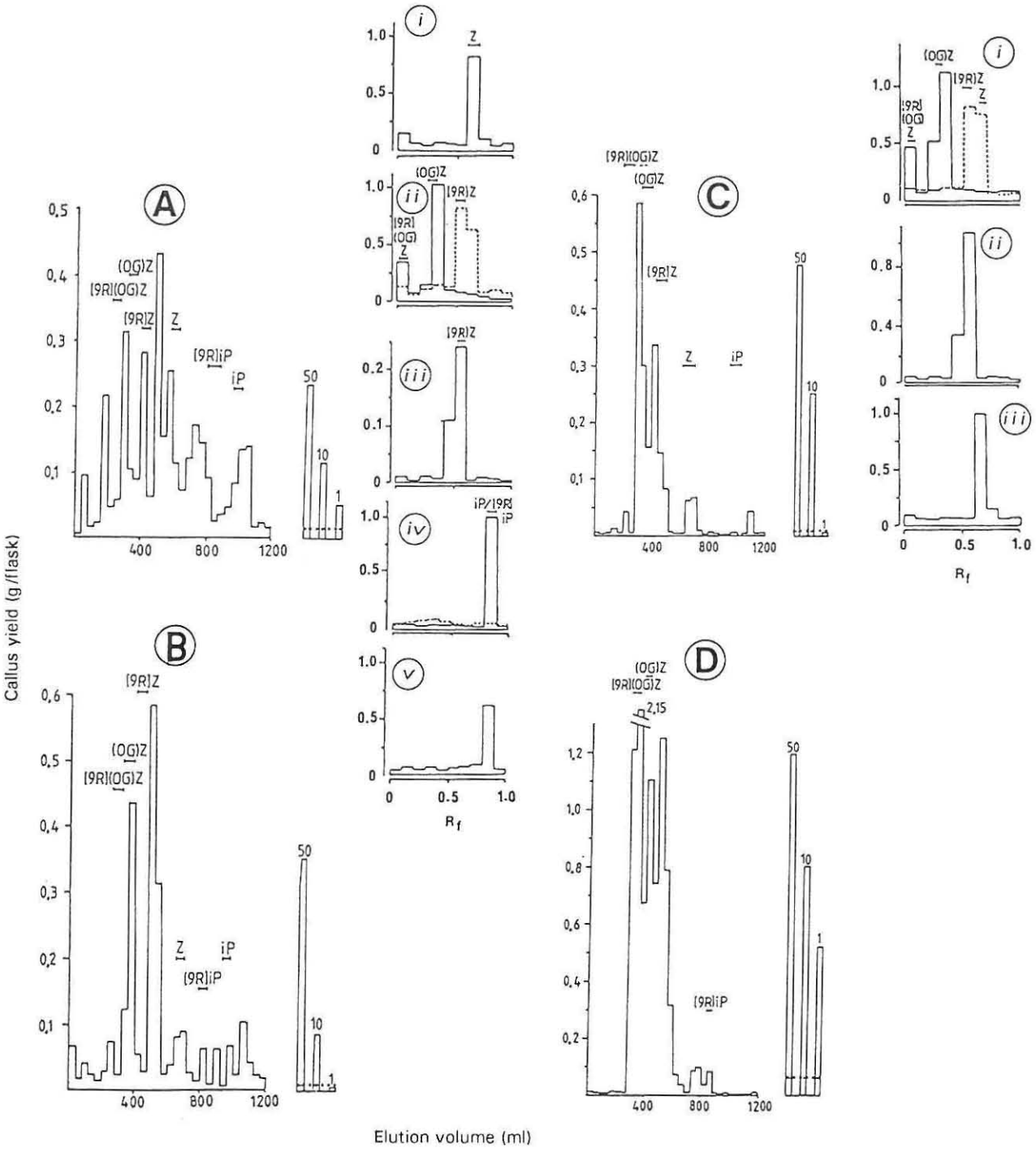

Figure 3 Cytokinin-like activity detected using the soybean callus bioassay after separation of ethanolic extracts of leaves of *Ginkgo biloba* collected on 12 May (A), 26 May (B), 9 June (C) and 25 June (D – post abscission), using Sephadex LH-20. Inserts A (i–v) and D (i–iii) represent biological activity in the soybean callus bioassay, measured after TLC separation of relevant peaks as described in text, using a solvent system of *n*-butanol:25% ammonium hydroxide: water (6:1:2). The dotted line represents activity detected after treatment with β-glucosidase prior to TLC analysis. Bars on the right represent activity detected with standards of 50, 10 and 1μg kinetin ml^{-1}. The dotted line at the base indicates the control callus yield.

opposed to its non-saturated relative (Figure 5). This was confirmed by the observation that, on treatment with β-glucosidase followed by $KMnO_4$ oxidation, activity remained, but shifted and subsequently co-eluted with DHZ. Additionally, although no [9R](OG) activity was detected in the untreated sample, compounds co-chromatographing with [9R]DHZ were detected after β-glucosidase hydrolysis. An unknown compound eluting at fraction 47 was also detected after this treatment. The activity recorded in this fraction therefore appears to be due to the presence of (OG)DHZ and possibly its ribosylated derivative with additional activity from unknown compounds. The third-largest peak of activity, eluting at 440 ml, co-eluted with authentic [9R]Z after both Sephadex and TLC separation [Figure 3A (iii)].

Eluting at 720–800 ml and 920–1120 ml was activity co-chromatographing with iP and [9R]iP respectively. Separation by TLC confirmed that these compounds had chromatographic properties similar to iP-like derivatives [Figure 3A (iv) & (v)].

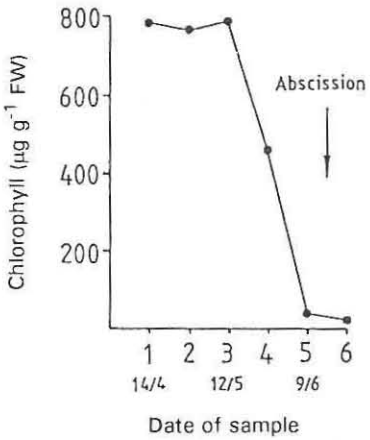

Figure 4 Chlorophyll concentrations in methanolic extracts of leaves of *Ginkgo biloba* from 14 March to after abscission in mid-June. Concentrations were calculated according to the equations of Lichtenthaler (1987).

Activity related to an unknown polar peak was also recorded at an elution volume of 200 ml in the mature leaf sample.

In order to investigate the cytokinins present in the mature leaves of *Ginkgo biloba* in more detail, analysis by HPLC was attempted. Unfortunately the decrease in activity after separation by Sephadex LH-20 and by HPLC made it difficult to detect the presence of certain compounds, particularly those co-chromatographing with the free base forms. Samples were therefore prepared for HPLC immediately after purification by paper chromatography. As the HPLC system used did not successfully resolve the free base Z from (OG)DHZ, the paper chromatograms were divided into regions of polar (R_f 0.0–0.5) and non-polar (R_f 0.5–1.0) activity before elution, as illustrated in Figure 6. This allows for the separation of the O-glucoside from the free base.

Separation of the polar fraction by HPLC confirmed the results obtained using separation by Sephadex LH-20 in that activity was found associated with (OG)DHZ, (OG)Z or [9G]Z, [9G]DHZ, [9R](OG)Z and to a lesser extent [9R](OG)DHZ (Figure 7A). Evidence of compounds co-chromatographing with [9R-MP]Z and [9R]Z were also detected. Treatment with β-glucosidase resulted in a shift of activity to co-elute mainly with [9R]Z, [9R]DHZ and an unknown compound eluting in fraction 46, whilst the activity associated with [9R-MP]Z remained unchanged (Figure 7B). β-Glucosidase treatment decreased the

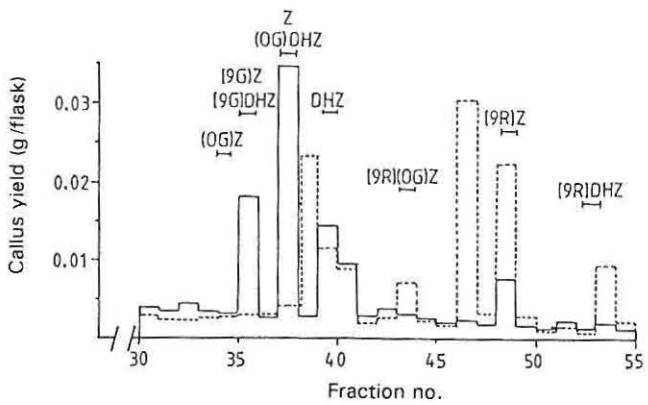

Figure 5 Cytokinin-like activity detected by the soybean callus bioassay after HPLC analysis of fractions co-eluting with (OG)Z on Sephadex LH-20. The dotted line represents activity present after treatment with β-glucosidase and $KMnO_4$.

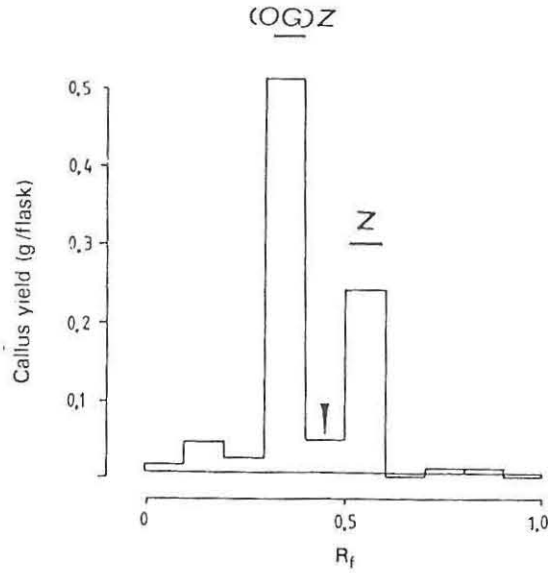

Figure 6 Cytokinin-like activity detected using the soybean callus bioassay after separation of ethanolic extracts of leaves of *Ginkgo biloba* by paper chromatography using a solvent system of isopropanol:25% ammonium hydroxide water (10:1:1). The arrow indicates the point of division into polar and non-polar activity for subsequent elution and analysis.

level of activity co-eluting with (OG)Z and the [9G] conjugates of Z and DHZ, thus indicating that this activity was mainly due to the former conjugate. The residual activity after this treatment does, however, indicate the presence of some [9G] conjugates.

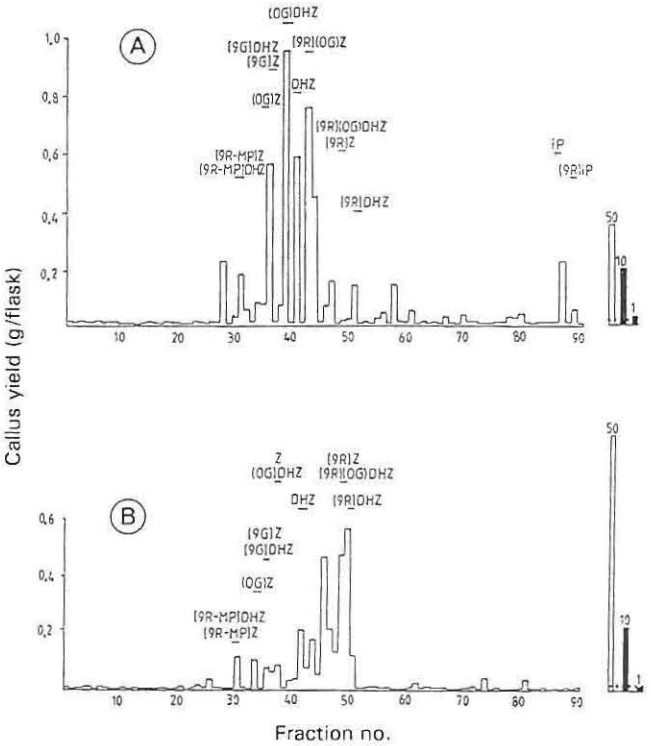

Figure 7 Cytokinin-like activity detected by the soybean callus bioassay after HPLC analysis of the polar fractions of ethanolic extracts of mature leaves of *Ginkgo biloba* untreated (A), and after treatment with β-glucosidase (B). Bars on the right represent activity detected with standards of 50, 10 and 1 µg ml^{-1} kinetin. The dotted line indicates the control callus yield.

The presence of iP derivatives detected after Sephadex LH-20 separations was confirmed using the HPLC system. Some activity was associated with DHZ in the non-treated polar fraction. Presence of DHZ may be due to hydrolysis of (OG)DHZ on extraction. This would not be detected with respect to Z, as activity co-eluting with this compound would be masked by (OG)DHZ activity in untreated polar fractions.

HPLC analysis of the non-polar fractions illustrated that the major free base in mature leaves of *Ginkgo biloba* co-eluted with authentic Z (Figure 8). Additionally, [9R]Z appears to be the dominant ribosyl derivative. A very large peak of activity was found to elute 1 min before authentic [9G]iP, and could possibly be due to the presence of other N-conjugated iP derivatives. Activity was also found associated with iP and its ribosylated derivative, as in the samples separated by Sephadex LH-20, but resolution of these two compounds was not successfully achieved using the present HPLC system.

Separation of ethanolic extracts of senesced and abscinded leaves of *Ginkgo biloba* by Sephadex LH-20 resulted in the detection of three major peaks of cell-dividing activity (Figure 3D). The largest, with an elution volume of 320–360 ml, co-eluted with (OG)Z and [9R](OG)Z. The possible presence of these derivatives was confirmed by TLC, as in the mature leaves [Figure 3D (i)]. Evidence of the presence of ribosylated forms of the free base was found, as a large peak of activity was detected at an elution volume of 440 ml. TLC analysis confirmed that this peak was due to compounds with similar chromatographic properties to [9R]Z [Figure 3D (ii)]. As in the mature leaves, a major peak of activity was detected with an elution volume of 520 ml. This did not co-elute with any cytokinin standards on Sephadex LH-20, but TLC analysis revealed that it has properties similar to those of the free bases Z and DHZ [Figure 3D (iii)].

As with the extracts from mature leaves of *Ginkgo biloba*, senescent leaf extracts were purified by paper chromatography and separated into polar and non-polar fractions prior to HPLC analysis. Separation of the polar fraction by HPLC resulted in the detection of six peaks of activity in the soybean callus bioassay (Figure 9A). The largest of these co-eluted with authentic DHZ.

Two smaller peaks of activity were found associated with authentic (OG)Z and (OG)DHZ. Some activity was also detected in the fraction co-eluting with [9R-MP]Z. Two unknown peaks of activity were found in fractions 19 and 45.

Separation of the polar fraction by HPLC generally showed very little cytokinin-like activity, with evidence of small amounts of activity co-eluting with [9R]DHZ and [9R]Z (Figure 9B).

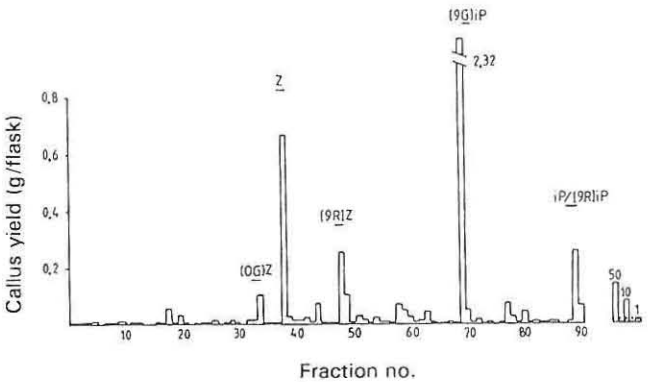

Figure 8 Cytokinin-like activity detected by the soybean callus bioassay after HPLC analysis of the non-polar fractions of ethanolic extracts of mature leaves of *Ginkgo biloba*. Bars on the right represent activity detected with standards of 50, 10 and 1 μg ml^{-1} kinetin. The dotted line indicates the control callus yield.

Additionally, activity was detected in fractions co-eluting with [9R-MP]iP and there was some evidence of the presence of iP-like derivatives.

Changes in endogenous cytokinins in leaves of *Salix babylonica* with foliar senescence

Ethanolic extracts of leaves of *Salix babylonica* showed both a qualitative and quantitative decrease in cytokinin content with leaf maturity (Figure 10). This was mainly observed as a decrease in the proportion of active compounds co-chromatographing with cytokinin O-glucosides. These compounds decreased markedly between 14 and 27 April. No activity was found at this elution volume by 12 May.

The main area of activity in this sample co-eluted with unknown polar compounds, although some activity was associated with the free base cytokinins of Z and iP, and their ribosides. This disappearance of O-glucoside activity coincided with the onset of senescence as measured in terms of chlorophyll loss (Figure 11). The dates of samples used in the analysis of endogenous cytokinins in *Salix babylonica* are different to those of *Ginkgo biloba*, as the former species senesced earlier in the year.

Separation of ethanolic extracts of mature leaves of *Salix babylonica* by Sephadex LH-20 revealed the presence of four major peaks of cytokinin-like activity. The largest of these co-eluted with authentic (OG)Z, and on TLC was found to consist of a major peak co-eluting with (OG)Z and two smaller peaks co-eluting with [9R](OG)Z and [9R]Z respectively [Figure 10A (i)]. The second-largest peak of activity eluted immediately after the passage of the void volume and therefore indicated the presence of very polar compounds with cell division-inducing activity. Eluting at 1 160 ml was another unknown compound active

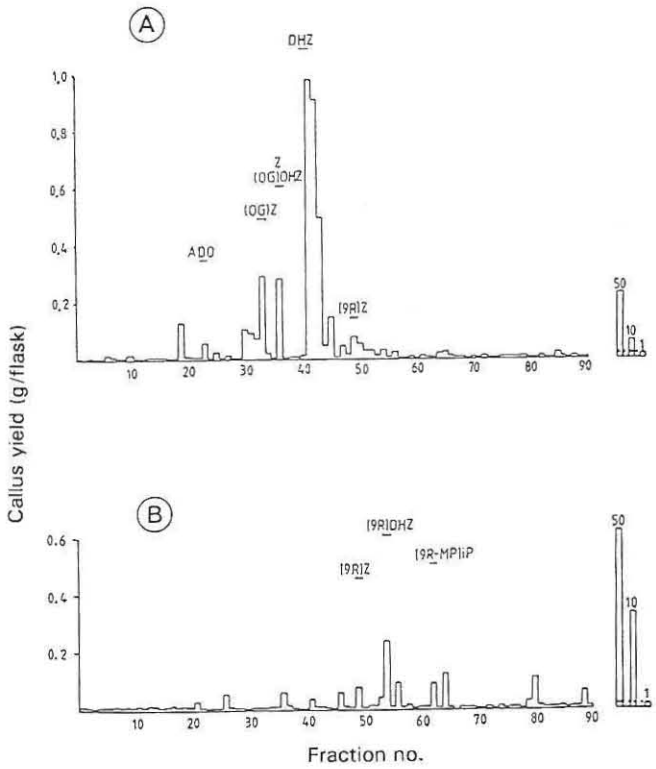

Figure 9 Cytokinin-like activity detected by the soybean callus bioassay after HPLC analysis of the polar (A) and non-polar (B) fractions of ethanolic extracts of senesced and abscinded leaves of *Ginkgo biloba*. Bars on the right represent activity detected with standards of 50, 10 and 1 μg ml^{-1} kinetin. The dotted line indicates the control callus yield.

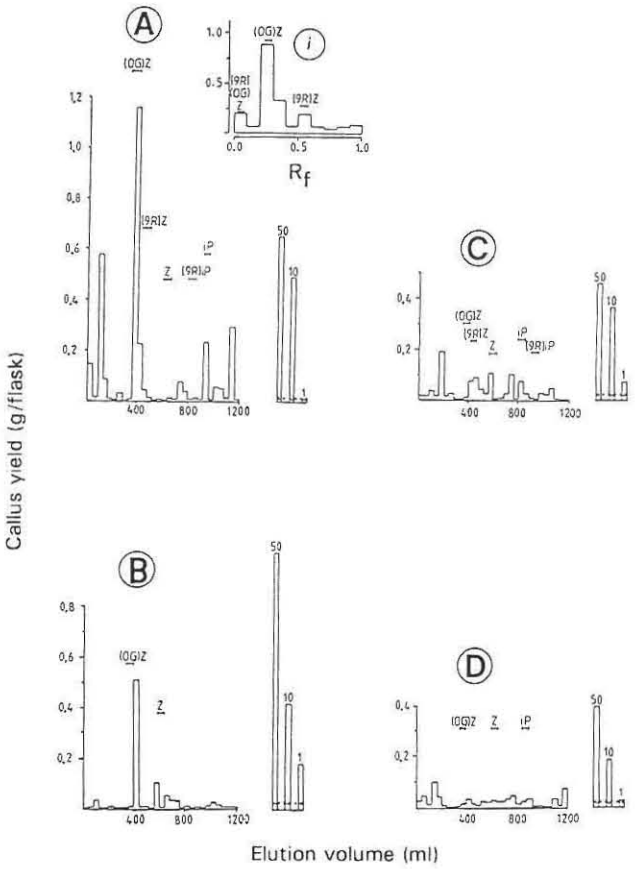

Figure 10 Cytokinin-like activity detected using the soybean callus bioassay after separation of ethanolic extracts of leaves of *Salix babylonica* collected on the 14 April (A), 27 April (B), 12 May (C) and 26 May (D), using Sephadex LH-20. Bars on the right represent activity detected with standards of 50, 10 and 1 μg kinetin ml^{-1}. The dotted line indicates the control callus yield.

in the soybean callus bioassay. The fourth-largest peak co-eluted with authentic iP, having an elution volume of 960 ml. Small peaks of activity were also recorded between the elution volumes of authentic Z and [9R]iP at 760 ml, and after iP at 1 080 ml.

Certain problems were encountered with the analysis of leaves of *Salix babylonica* by HPLC, due to the low concentrations of cytokinin present in this tissue as compared to that of *Ginkgo biloba*. The most effective method of analysis was to subject the whole sample to HPLC separation, without division into polar and non-polar fractions. Thus after an initial purification by paper chromatography, complete ethanolic extracts of mature *Salix babylonica* leaves were separated and analyzed using reverse-phase HPLC (Figure 12). Seven peaks of activity were recorded, the largest co-eluting with (OG)DHZ and Z. The second largest peak of activity was associated with unknown compound/s present in fraction 44.

As in the samples separated by Sephadex LH-20, the presence of a polar compound was detected in fractions 19–20, eluting just before authentic adenosine. In addition to this, activity was also recorded just prior to the retention time of [9R-MP]Z and [9R-MP]DHZ. It is therefore possible that the large polar peak detected after separation by Sephadex LH-20 may be due to the presence of these two polar compounds separated by HPLC. A fairly large peak of activity was found associated with [9R]Z, [9R](OG)DHZ and [9R]DHZ. Complete resolution of this activity into discrete peaks, however, was not possible.

Although HPLC analysis of extracts of senescing leaves of *Salix babylonica* was carried out, these results are not illustrated.

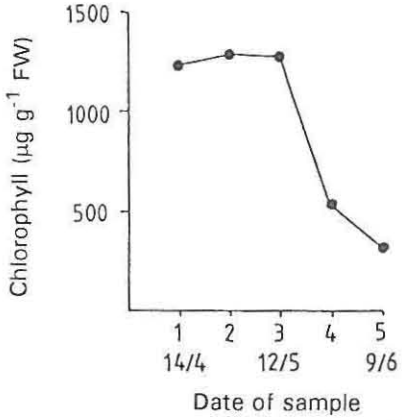

Figure 11 Chlorophyll concentrations in methanolic extracts of leaves of *Salix babylonica* from 14 March to abscission in early June. Concentrations were calculated according to the equations of Lichtenthaler (1987).

No peaks of activity were recorded at all, therefore confirming the results achieved after separation by Sephadex LH-20.

Discussion

The most common pattern of change in endogenous cytokinins with leaf maturity and age is an increase in the proportion of O-glucosyl derivatives (van Staden *et al.* 1988). As these compounds accumulate in mature and senescing leaves, it is possible that their concentrations, as opposed to those of active free base forms, may be instrumental in triggering the onset of senescent processes. After investigating the change in endogenous cytokinin content of leaves of both *Salix babylonica* and *Ginkgo biloba*, however, this study demonstrates that such generalizations as to the control of deciduous leaf senescence by cytokinins may be both simplistic and misleading. As these two species differed so markedly in their change in foliar cytokinin content at senescence, it would appear that different mechanisms may govern the control of this syndrome in different species. Therefore, as both these species exhibit deciduous senescence, the mechanism of control is not necessarily related to the form of senescence.

The pattern of change in foliar cytokinin content with the onset of leaf senescence in *Salix babylonica* was found to be consistent with the hypothesis that a decrease in the concentration of these hormones may initiate the commencement of degradative reactions. A change in both the free base, mainly iP derivatives, and O-glucoside derivatives was observed prior to any change in

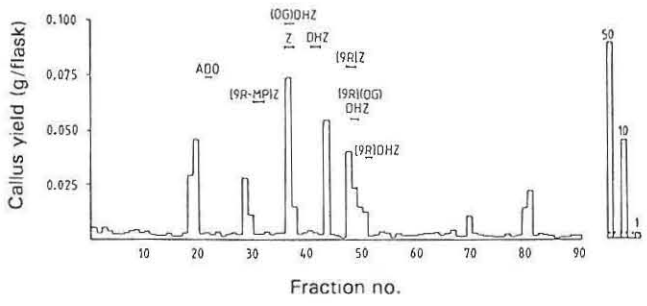

Figure 12 Cytokinin-like activity detected by the soybean callus bioassay following HPLC analysis of ethanolic extracts of mature leaves of *Salix babylonica* after purification by paper chromatography. Bars on the right represent activity detected with standards of 50, 10 and 1 μg ml^{-1} kinetin. The dotted line indicates the control callus yield.

chlorophyll concentration. It is therefore possible that this decrease in cytokinin activity may be instrumental in initiating the process of chlorophyll loss. Comparison of these results with those of van Staden (1977), however, reveals some contradictions. Although the two studies report remarkably similar results for leaves collected during April, van Staden (1977) presents evidence that a large amount of cytokinin activity, co-chromatographing with both the O-glucoside and free base, is still present in leaves during the month of May. This would suggest that a decrease in cytokinin content does not precede the onset of senescence, and therefore does not play any role in its initiation. Van Staden (1977), however, did not include any empirical measure of the developmental state of the leaf with respect to the progress of senescence, only stating that they appeared to be yellowing in early April. Additionally, the work does not include a measure of foliar cytokinin activity immediately prior to abscission. It is therefore possible that, due to different prevailing environmental conditions, senescence commenced earlier during the sampling period of the current investigations, and the monthly comparative samples do not represent leaves of the same developmental stage.

Despite the differences between these two studies with respect to later samples, both report a very similar cytokinin complement in mature leaves of *Salix babylonic*a. The major peak of cytokinin activity was associated with O-glucoside and possibly O-glucoside riboside derivatives of DHZ. No activity was found to co-elute with free zeatin or its dihydro derivative in this study or that of van Staden (1977). Free base cytokinins therefore appeared to be in the form of iP. This demonstrates that O-glucosylation of Z and DHZ and their derivatives is the major form of metabolism in mature leaves of *Salix babylonica*, and must take place very rapidly after supply of the free base via the xylem, or biosynthesis *in situ*. N-glucosylation of iP derivatives does not appear to be an important metabolic pathway, although some evidence of these compounds was detected.

In comparing the foliar cytokinin complement of *Salix babylonica* to that of *Ginkgo biloba*, the first factor to take into consideration is the remarkably high levels of activity in the latter species. Additionally, *Ginkgo biloba* contained a far greater variety of cytokinins than *Salix babylonica*, with the presence of many unknown compounds exhibiting cell-dividing activity. As these two species are evolutionarily far removed from each other it is not surprising that there may be great differences in the biochemical composition of their leaf tissue. The large amount of cytokinin-like activity within the leaves of *Ginkgo biloba*, however, may not be related to the fact that it is a gymnosperm. Van Staden (1978) investigated the cytokinin content of leaves of four different gymnosperms. The cytokinin content of *Ginkgo biloba* was markedly higher than that of any of the other species tested, showing a predominance of O-glycoside derivatives. It is possible that the high levels of cytokinins within the leaf tissue of *Ginkgo biloba*, compared to other gymnosperms and the angiosperm *Salix babylonica*, may be related to the development of the deciduous habit in a relatively primitive species.

In direct contrast to the situation in *Salix babylonica*, cytokinin O-glucoside content in leaves of *Ginkgo biloba* was found to increase with leaf senescence right up until abscission. This increase coincided with the onset of chlorophyll loss, and continued throughout the senescence process. Additionally, evidence appeared to suggest that there was a shift from a predominance of riboside derivatives in leaves prior to senescence, to a predominance of O-glucoside and O-glucoside riboside derivatives in senescent leaves. Letham *et al.* (1976) report a similar situation in leaves of *Populus nigra*. ^{3}[H]Z fed to mature leaves of this species was rapidly metabolized to [9R](OG)Z and [9R)(OG) DHZ. On leaf senescence, however, these compounds were deribosylated to produce O-glucoside derivatives. In addition to these changes, the current study demonstrates that a decrease in activity co-chromatographing with Z or DHZ and iP slightly preceded chlorophyll loss, with only a residual amount of activity remaining prior to and after leaf abscission. These results are therefore consistent with the hypothesis that a shift in the flux between free base and O-glucoside may initiate the onset of senescence, but demonstrates that ribosyl and O-glucoside ribosyl derivatives may also be involved.

The hypothesis that an accumulation of O-glucoside derivatives may actually trigger the onset of senescence is dependent on one major factor – the relative activity of these compounds in biological systems. For O-glucosylation to act by decreasing the free base pool within the plant cell, the derivatives would themselves have to be biologically inactive. Evidence has, however, been presented that not only are cytokinin O-glucosides active in both the soybean callus (van Staden & Papaphilippou 1977; Letham *et al.* 1983; van Staden & Drewes 1991) and oat leaf senescence bioassays (Letham *et al.* 1983), but this activity may exceed that of the free base or 'active' form. These observations do not take into consideration one vital issue – the natural compartmentation of different cytokinin groups within the plant cell. Analysis of biological activity by the bioassay method involves application of a test compound and observation of the resultant response. The activity of an exogenous compound may not necessarily be comparable to that of the same compound in its endogenous situation. This is due to the fact that exogenous application results in contact with membranes and enzyme systems otherwise not encountered by that substance (McGaw & Horgan 1985). The compound in its correct localization within the cell may therefore have a specific effect at a specific point in a cascade of events, which is intimately related to its intracellular positioning. Therefore, the biological activity, or inactivity, of cytokinin O-glucosides with respect to senescence may be a function of their compartmentation within the cell with respect to the enzyme systems involved in their hydrolysis (Palmer *et al.* 1981a, 1981b). Evidence to support this hypothesis has been supplied by Fuseder & Ziegler (1988), who investigated the compartmentation of exogenously supplied [^{3}H]DHZ in suspension cultures of *Chenopodium rubrum*. These workers demonstrated that the major metabolites of [^{3}H]DHZ, [9R](OG)DHZ and (OG)DHZ, were located exclusively in the cell vacuole, whereas DHZ and [9R]DHZ were localized predominantly outside the vacuole. Glucoside conjugates of other plant growth substances such as ABA (Bray & Zeevaart 1985; Lehumann & Glund 1986), GA_1 (Garcia-Martinez *et al.* 1981) and the synthetic auxin 2,4-dichlorophenoxyacetic acid (Schmitt & Sanderman 1982) have also been shown to be specifically compartmented within the plant vacuole. If this is considered in context with the process of senescence, many substances are deposited in the vacuole prior to and during the course of degradative processes (Thimann 1987). The best example of this is the vacuolar sequestration of waste products of chlorophyll degradation reported to occur during senescence of barley leaves (Matile *et al.* 1988). It is therefore possible that the accumulation of O-glucoside derivatives that has been shown to take place in senescing leaves of *Ginkgo biloba* is due to a preferential sequestration within the vacuole with leaf age. As Thimann (1987) has hypothesized that the onset of intracellular degradation is marked by an efflux of hydrolytic and proteolytic enzymes from the vacuole, the putative intracellular localization of cytokinin O-glucosides introduces the possibility that these substances may be involved in the mechanism of this process.

Subsequent to their work on investigating the intracellular localization of cytokinin O-glucosides, Fuseder *et al.* (1989) demonstrated that the vacuolar sequestration of these compounds

is a reversible process in suspension cultures of *Chenopodium rubrum*. As vacuolar O-glucosides were converted preferentially to polar compounds of as yet unknown nature, the authors concluded that they may serve to maintain a small, but more or less constant pool of extra-vacuolar (presumably cytosolic) aglycones. This, therefore, introduces the question as to the role these compounds play in plant systems, especially that of senescing leaves. The reversible sequestration of cytokinin O-glucosides into the vacuole of cells of *Chenopodium rubrum* obviously indicates a possible storage role in this system. As previously stated, transport of glucosides from the leaf prior to abscission would be necessary if a storage role was to be demonstrated in deciduous trees. The dramatic decrease in O-glucoside content of *Salix babylonica* leaves prior to abscission, as demonstrated in this study, suggests that such transport may occur in the aforementioned species. Consideration of the situation in *Ginkgo biloba*, however, is more complex. As the leaves of this species contain a large amount of O-glucoside activity after abscission, it appears unlikely that export of the conjugates does take place. Due to the high levels of activity present in these leaves during senescence, the detection of export of small quantities of cytokinin O-glucosides to persisting organs would be difficult. It is therefore possible that a certain amount of O-glucosides are exported until a threshold level is reached, after which the remaining foliar concentrations represent waste products lost on abscission.

This study shows that changes in cytokinin content in leaves of *Salix babylonica* and *Ginkgo biloba* may be related to the onset of chlorophyll loss. Whether these changes are involved in the mechanism of action controlling the onset of senescence remains to be conclusively elucidated. It has been shown, however, that different mechanisms must exist in different species and that the method of control is not related to the form of senescence displayed by the plant under question. Evidence has been presented that cytokinin O-glucosides may be exported from the leaves of *Salix babylonica* prior to abscission and may therefore represent putative storage compounds. Although a large amount of O-glucosides are present in leaves of *Ginkgo biloba* subsequent to abscission, this does not necessarily mean that they are completely lost to the plant. A greater understanding into the importance of cytokinins in controlling the onset of deciduous senescence would therefore be achieved by a more detailed investigation into the fluxes between the different pools of cytokinin within the cell with respect to specific constituent processes of the senescence syndrome. More sensitive methods of cytokinin analysis, such as radioimmunoassay, in conjunction with studies on intracellular localization, may achieve this aim.

Acknowledgements

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